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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Nerve agent exposure inhibits acetylcholinesterase, leading to increased and prolonged stimulation of acetylcholine receptors. Common consequences of this cholinergic crisis include seizure activity, neuronal damage and behavioral deficits. The paucity of research directed toward the infant/juvenile population has raised concern because of the unique vulnerabilities of children. In the current study, male and female rats exposed to sarin (GB) were evaluated on tests of spatial memory, locomotor activity and vestibular motor function, as well as neuropathology. Similar to our adult model, we found that juvenile rats exposed to GB exhibited deficits in vestibular motor function for up to 1 week and cognitive deficits in the Morris water maze at 3 weeks post-exposure. In addition, extensive neuropathology and spontaneous recurrent seizures (SRS) were observed. The current results demonstrate the vulnerability of a juvenile population to motor impairments, cognitive deficits, neuropathology and SRS following exposure to GB.

15. SUBJECT TERMS

Nerve agent, sarin, development, juvenile, rat

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Introduction -

There is limited research on the developmental toxicity associated with exposure to nerve agents. The majority of research has focused on the toxic effects of nerve agents in adult animal models. In the event of a mass casualty situation involving the release of nerve agents like sarin (GB), infants, children and adolescents are likely to be exposed. It is critical to evaluate whether countermeasures that are effective against nerve agents in adult animals will also be efficacious in young animals. However, prior to the evaluation of countermeasures, there is a need to develop standardized models of nerve agent exposure in young animals. To further those efforts, this project was undertaken to characterize the behavioral, physiological and pathological effects of sarin exposure in rats at various time points in their development.

Keywords -

Nerve agent, sarin, development, juvenile, rat

Overall Project Summary -

Characterization of the behavioral effects associated with sarin exposure during development

Methods

Male and female rats received a subcutaneous (sc) injection of saline (SAL) or one of three doses of GB (0.6, 0.8 or 1.0 LD_{50}; Table 1) on postnatal day (PND) 7, 21 or 42. PND 42 rats were surgically implanted with telemetry receivers (F40-EET; Data Sciences International, St. Paul, MN) approximately 1 week prior to exposures. Toxic signs were continuously observed for 1-2 hr post-exposure, and the rats were weighed daily (Monday-Friday). Rats were evaluated on a battery of behavioral tasks (Table 2) before being perfused on post-exposure day (PED) 32. Brains were removed and sent to FD Neurotechnologies, Inc. (Columbia, MD) for processing. An observer blind to the treatment groups quantified the amount of NeuN and GFAP-stained cells in brain regions known to be sensitive to nerve agents (e.g. amygdala, hippocampus, piriform cortex and thalamus).

Table 1. 24 h LD_{50} Values

Age Group	Males	Females
PND 7	50.0 µg/kg	51.7 µg/kg
PND 21	93.0 µg/kg	92.2 µg/kg
PND 42	212.1 µg/kg	233.0 µg/kg

Table 2. Behavioral Tasks

Behavioral Task	Cognitive Measure	Post-Exposure Day (PED)
Balance Beam, Runway	Motor coordination, gait	0, 1, 7
Figure-8 Maze	Motor activity	0, 1, 7
Morris Water Maze	Spatial memory and learning	22-25

Note: PND 7 rats were only tested on the Morris water maze.

In addition, a subset of male rats implanted with jugular catheters were exposed to 1.0 LD_{50} GB on PND 42. Blood was collected at various time points after exposure and analyzed for cardiac troponin (cTnI) levels. At 72 hr post-exposure, the rats were euthanized and their hearts removed for pathology.

Results

No differences were observed between male and female rats on any endpoint; thus, their data was combined.

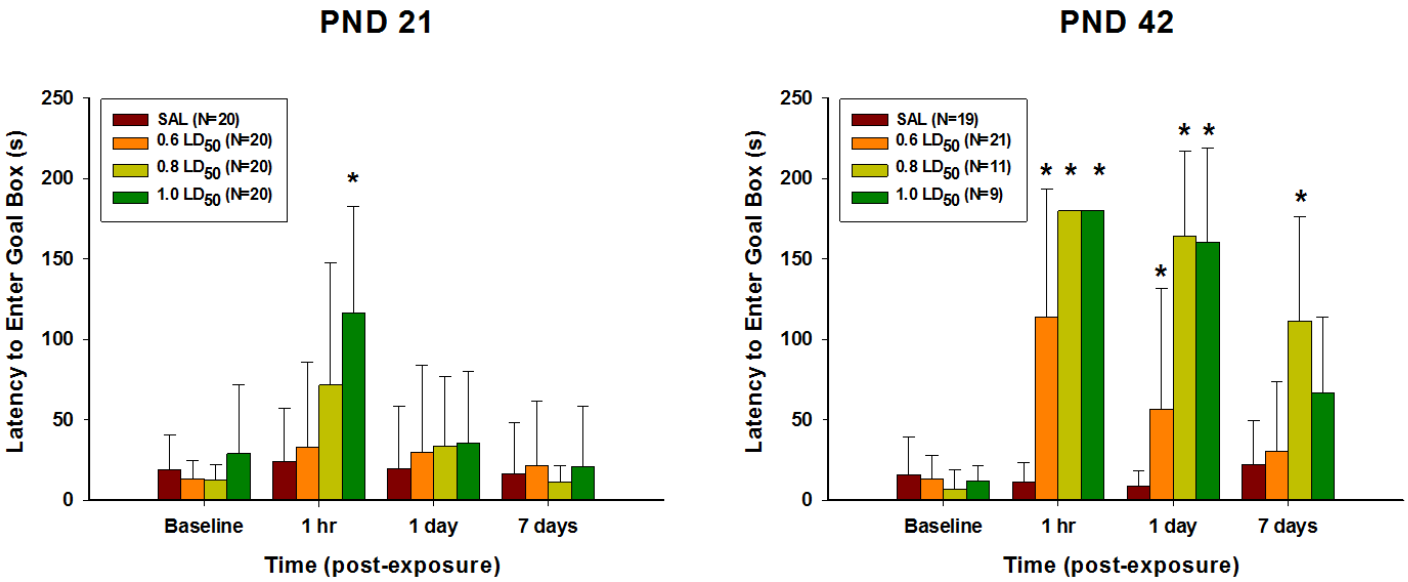

Figure 1. Latency to enter goal box (mean ± SD) on a balance beam task at 1 hr, 1 and 7 days following sc exposure to GB on PND 21 (left) or 42 (right). Compared to controls, rats exposed to 1.0 LD_{50} GB on PND 21 took significantly (*, p<.05) longer to reach the goal box of the balance beam at the 1 hr time point. Rats exposed to 1.0 LD_{50} GD on PND 42 took significantly longer to reach the goal box at both the 1 hr and 1 day time points.

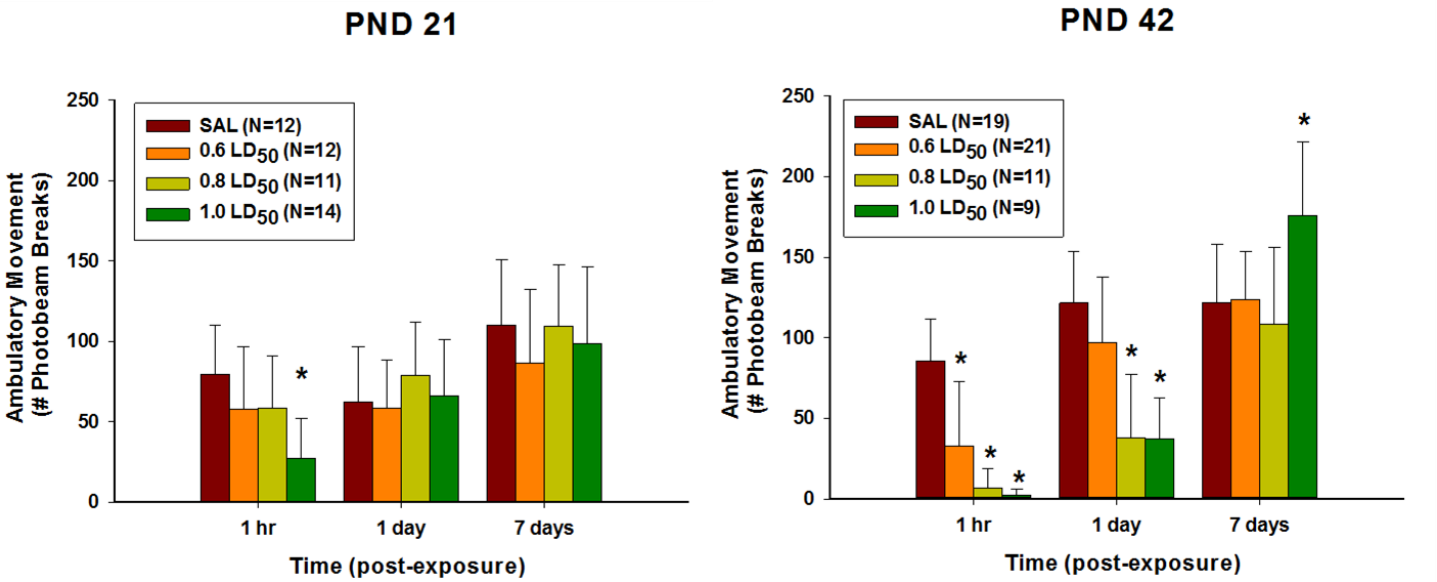

Figure 2. Ambulatory movements (mean ± SD) in the Figure-8 maze at 1 hr, 1 and 7 days following sc exposure to GB on PND 21 (left) or 42 (right). Compared to controls, rats exposed to 1.0 LD_{50} GB on PND 21 made significantly (*, p<.05) fewer movements in the Figure-8 maze at the 1 hr time point. Rats exposed to 1.0 LD_{50} GD on PND 42 made significantly fewer movements at both the 1 hr and 1 day time points and more movements at the 7 day time point.

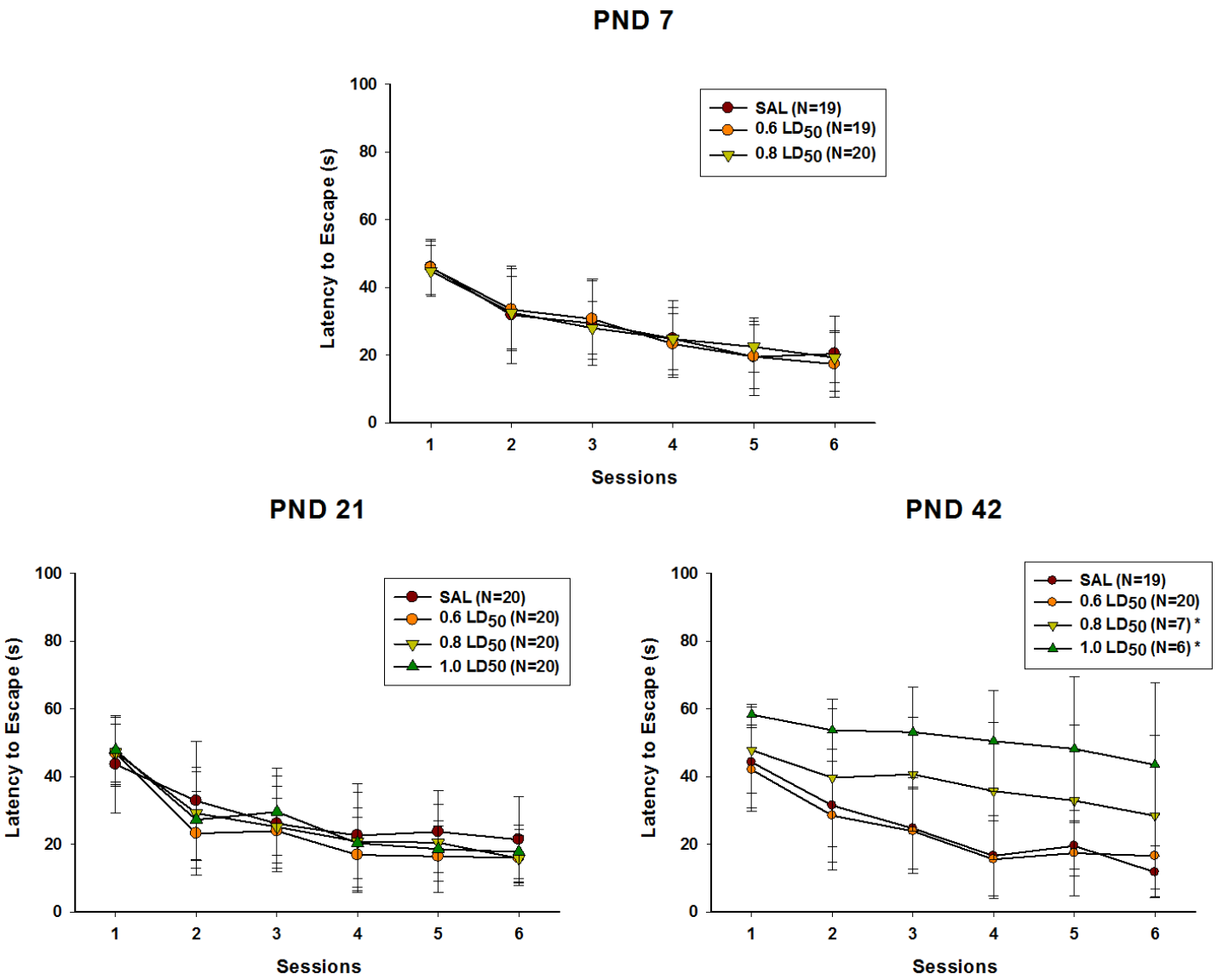

Figure 3. Latency to escape in the Morris water maze, which was conducted 3 weeks following sc exposure to GB on PND 7 (top), 21 (left) or 42 (right). Each data point represents the mean ± SD. Compared to controls, rats exposed to 0.8 or 1.0 LD_{50} GB on PND 42 were significantly (*, p<.05) impaired on the water maze. In addition, 8 rats had to be rescued from the pool due to convulsions and were unable to complete this task. No effects were observed with rats exposed to GD on PND 7 or 21.

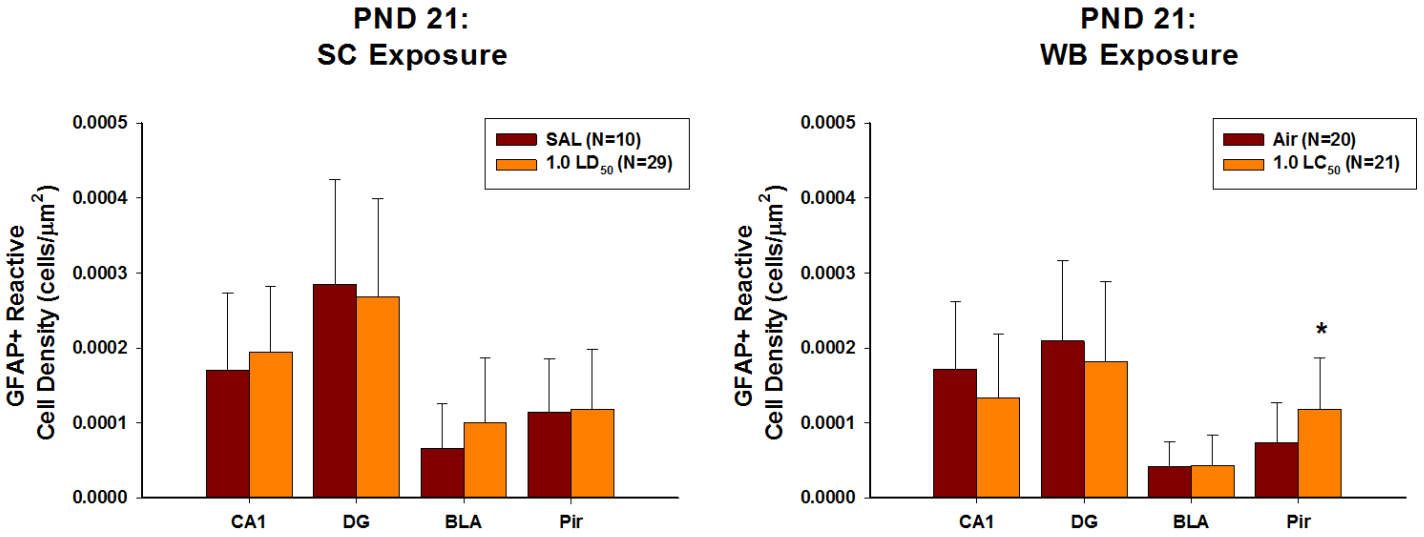

Figure 4. Density (mean ± SD) of GFAP+ reactive cells in various brain regions (CA1 region of the hippocampus, CA1; dentate gyrus, DG; basolateral amygdala, BLA; and piriform cortex, Pir) of rats exposed to GB via the subcutaneous (left) or whole-body (WB; right) route on PND 21. No differences were observed at 1 month post-exposure in the brains of rats sc exposed to 1.0 LD_{50} GB. However, a significant (*, p<.05) decrease in the number of GFAP+ neurons was observed at 1 year post-exposure in the brains of rats exposed to 1.0 LC_{50} GB via their whole bodies.

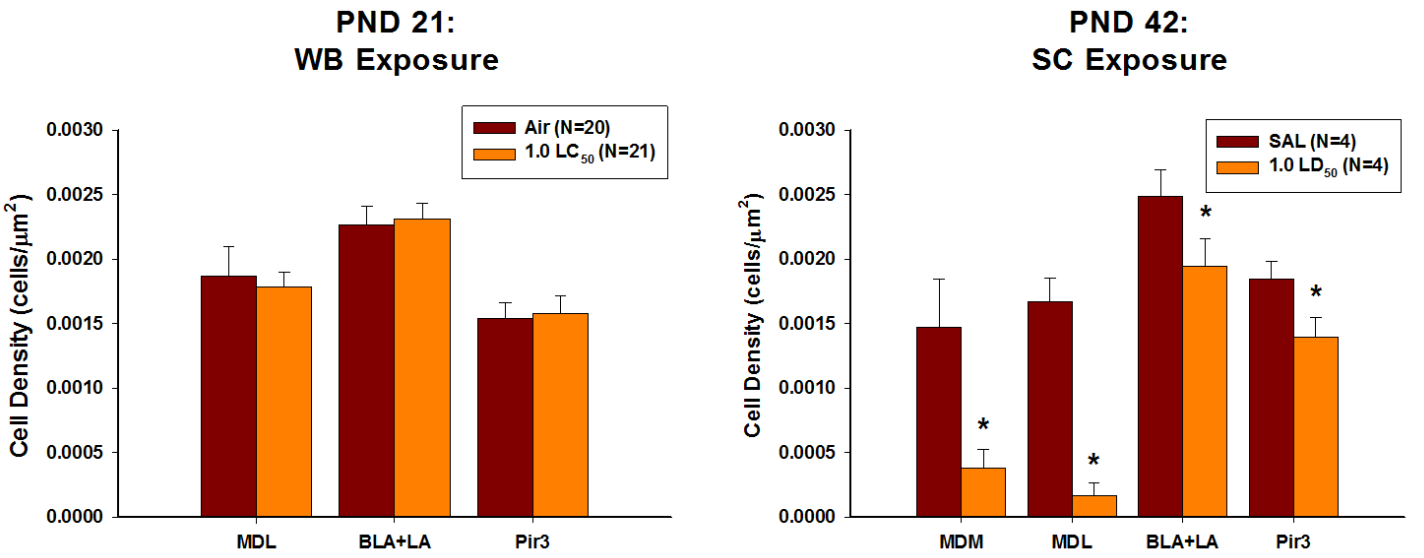

Figure 5. Density (mean ± SD) of NeuN-stained cells in various brain regions (basolateral and lateral amygdala, BLA+LA; layer 3 of the piriform cortex, Pir3; mediodorsal thalamic nucleus, lateral part, MDL; and mediodorsal thalamic nucleus, medial part, MDM) of rats exposed to GB via the whole-body route on PND 21 (left) or the subcutaneous route on PND 42 (right). Compared to controls, no differences were observed at 1 year post-exposure in the brains of rats exposed to 1.0 LC_{50} GB via their whole-bodies. At 1 month post-exposure, the density of new cells was significantly (*, p<.05) lower in the brains of rats exposed to 1.0 LD_{50} GB on PND 42.

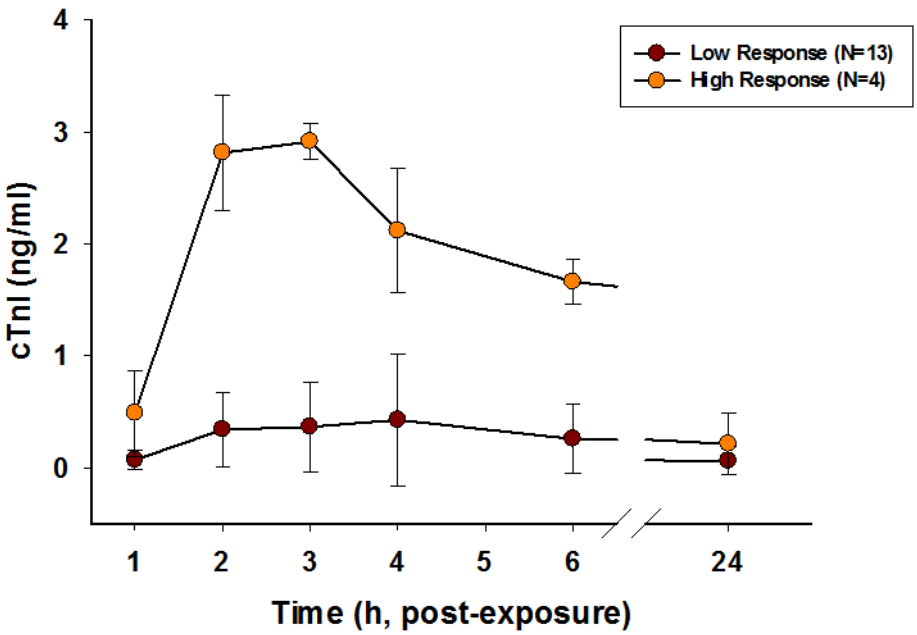

Figure 6. Levels of cardiac troponin (mean ± SD) in the blood of PND 42 rats sc exposed to 1.0 LD_{50} GB. Troponin levels peaked within a few (2-4 hr) of exposure before returning to baseline levels at 24 hr. Rats with peak troponin levels greater than 1 ng/ml were classified as high responders, whereas the remaining rats were classified as low responders (peak troponin levels were less than 1 ng/ml). A board-certified pathologist examined the hearts of these animals and determined that there was no evidence of myocardial damage.

Analysis of seizure activity and dendritic spine density following exposure to sarin during puberty

Methods

Female rats were surgically implanted with telemetry transmitters and sc exposed to SAL or one of two doses of GB (0.6 or 1.0 LD_{50}; Table 1) on PND 42. The rats were deeply anesthetized with a pentobarbital-based solution at 1, 6, 24 or 72 hr post-exposure, and the whole brains were removed and immersion-fixed in a Golgi stain (FD Neurotechnologies, Inc., Columbia, MD). Dendritic spine density in CA1 and BLA was quantified by Sinq Systems, Inc. (Silver Spring, MD).

Preparation of rat brains for Golgi-Cox staining: Upon euthanasia, the brain of each animal was carefully removed from the skull. The cerebrum was blocked coronally through the area between Bregma 1.20 mm and -0.84 mm (The Rat Brain in Stereotaxic Coordinates by Paxinos & Watson, 2007). The tissue blocks were rinsed quickly but gently with Milli-Q water to remove excessive blood and then immersed in the impregnation solution. The brains were store at room temperature in the impregnation solution for up to 2 weeks before being shipped to FD Neurotechnologies, Inc. for further processing using their FD Rapid Golgistain™ kit.

EEG analyses: Full-power spectral analysis of EEG, identification of epileptiform activity and other EEG anomalies were analyzed according to the methods described previously in de Araujo Furtado et al. (2009). EEG seizures were confirmed through visual screening and characterized by sustained frequencies and the value of the most prominent frequencies in Hz (e.g. the highest power calculated by MatLab program in µV2/Hz).

Dendritic spine density analyses: Dendritic spines (Figure 7) were quantified by Sinq Systems, Inc., using measures of spine density, percentage of each type of spine (e.g. mushroom, thin and stubby), branches/Golgi+ neurons and lengths of dendrites/Golgi+ neurons in CA1 and BLA.

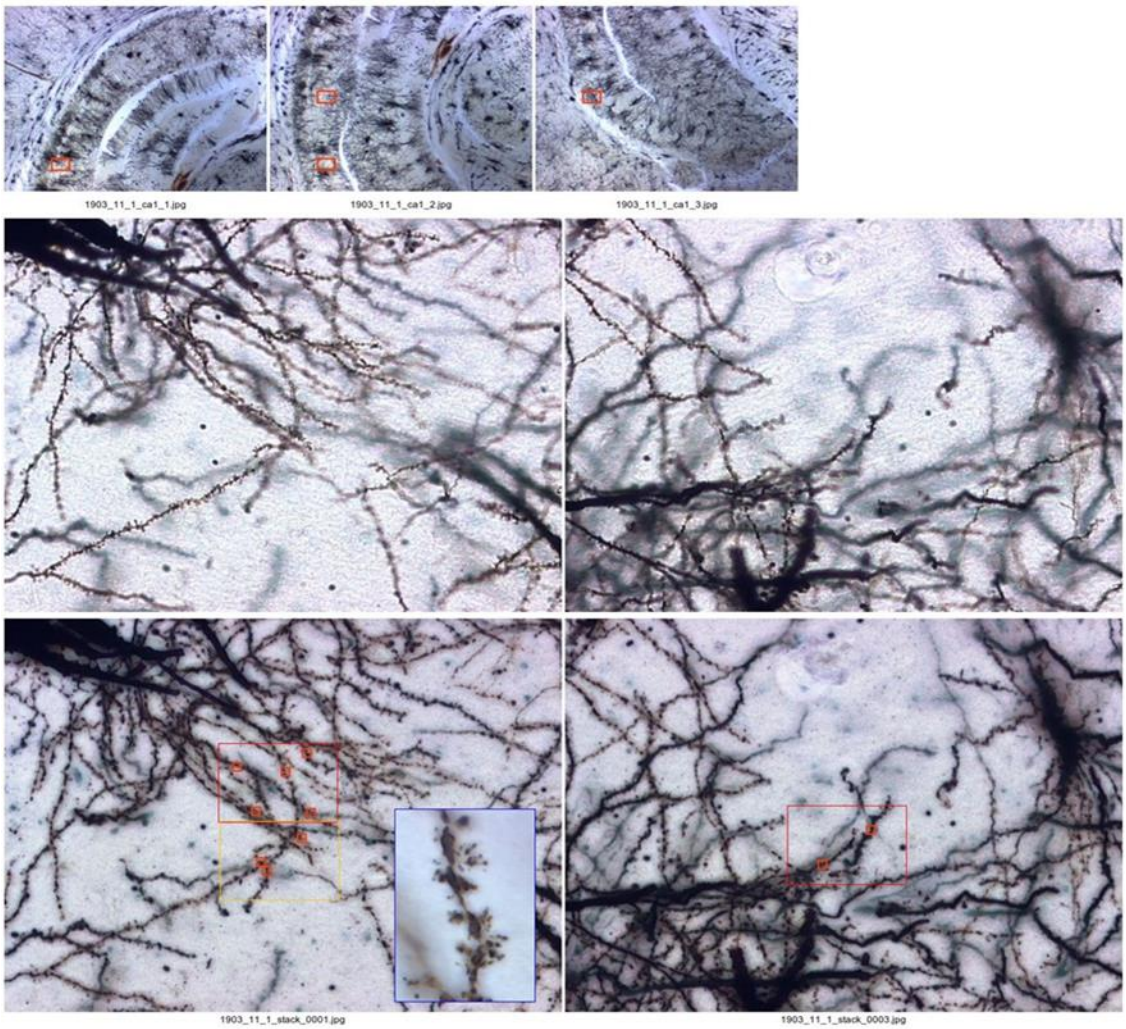

Figure 7. Representative figures of the dendritic spines that were quantified by Sinq Systems, Inc.

Results

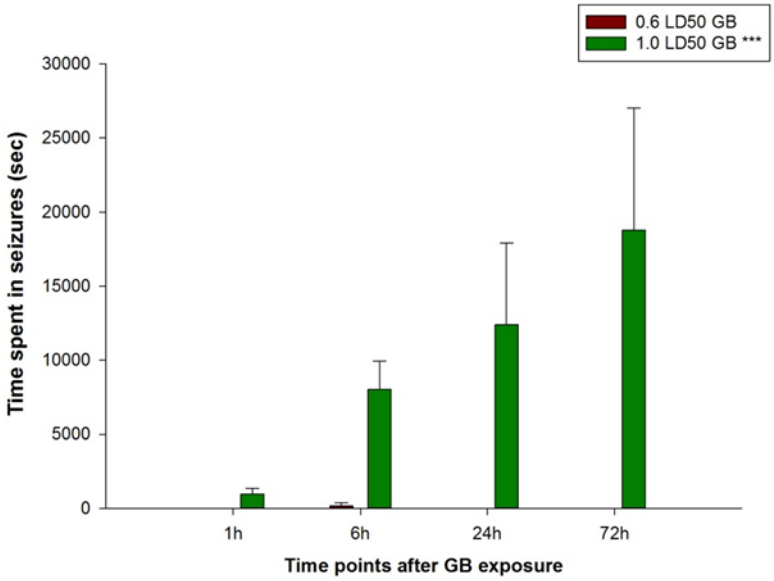

Figure 8. Time spent in seizures (mean ± SEM) for rats sc exposed to GB on PND 42 and euthanized at 1, 6, 24 or 72 h post-exposure. Only rats exposed to 1.0 LD_{50} GB developed seizures.

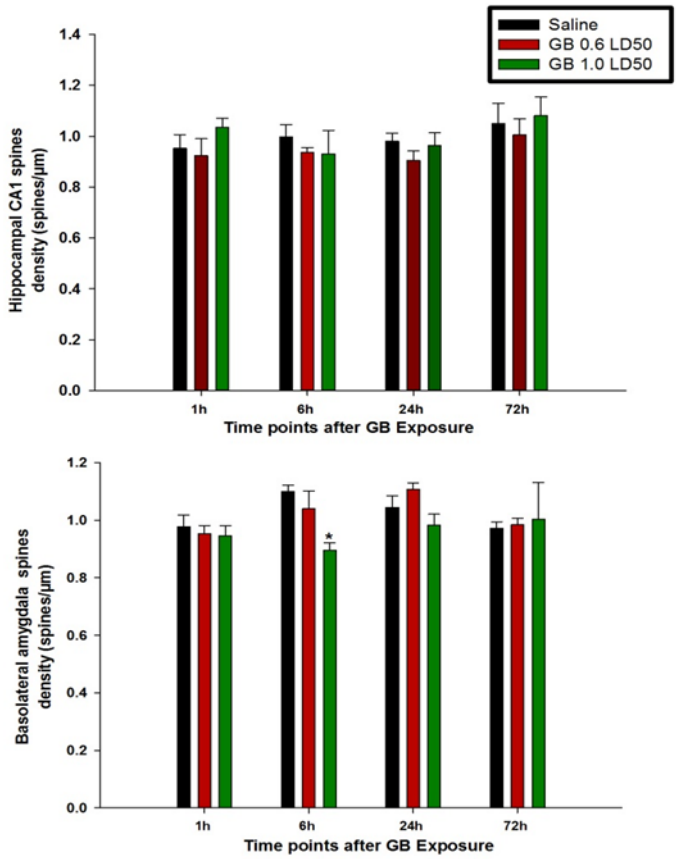

Figure 9. Dendritic spine density (mean ± SEM) in the CA1 (A) and BLA (B) of rats sc exposed to GB on PND 42 and euthanized at 1, 6, 24 or 72 h post-exposure. Dendritic spine density was significantly (*, p<.05) decreased at the 6 h time point in the BLA of rats exposed to 1.0 LD_{50} GB. In addition, each type of dendritic spine formation in the CA1 and BLA of rats sc exposed to GB on PND 42 and euthanized at 1, 6, 24 or 72 h post-exposure was evaluated (data not shown). Signficantly (*, p<.05) fewer stubby spines were observed at the 72 h time point in the CA1 of rats exposed to 0.6 LD_{50} GB and there was a trend for fewer mushroom spines in the CA1 and BLA of rats exposed to 1.0 LD_{50} GB.

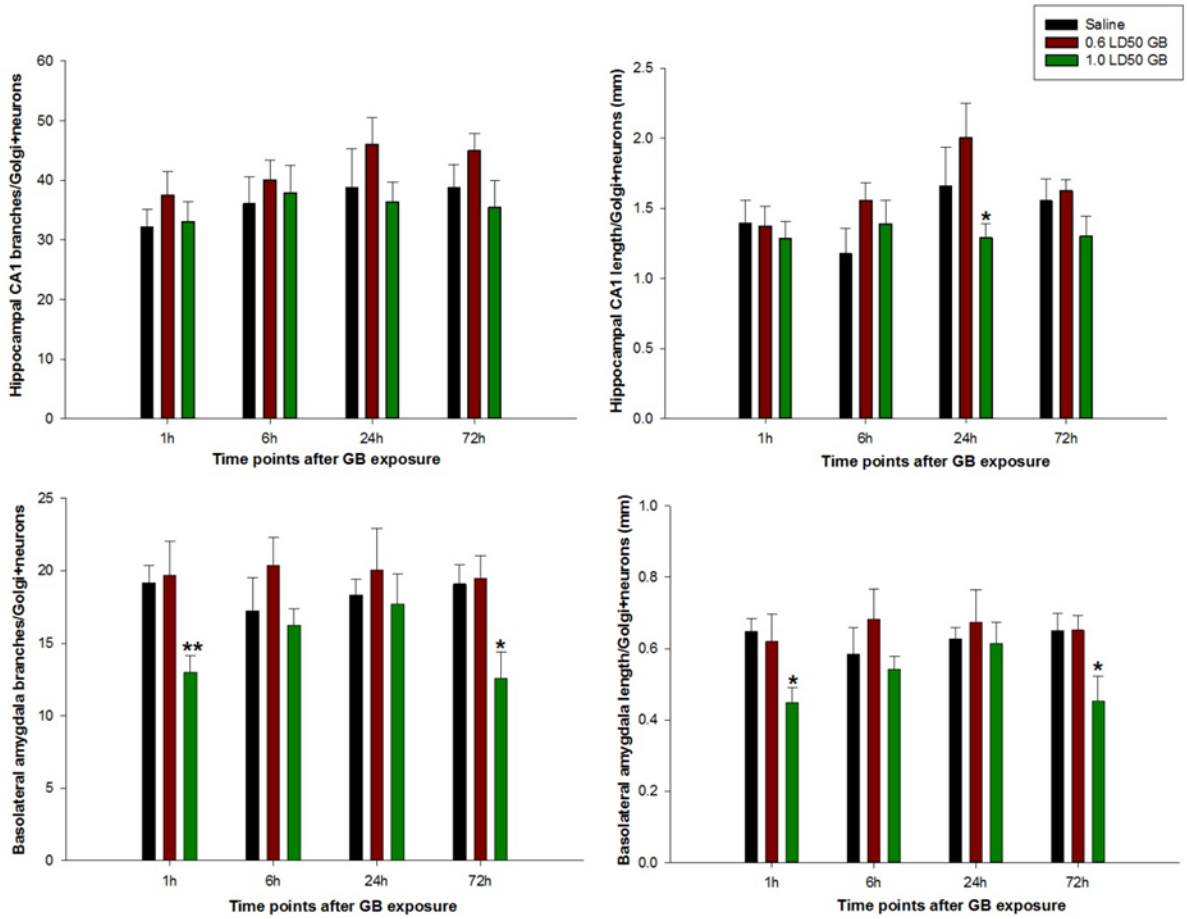

Figure 10. Number of branches (mean ± SEM) per Golgi+ neuron in the CA1 (A) and BLA (B), as well as the length (mean ± SEM) of each dendrite in the CA1 (C) and BLA (D), of rats sc exposed to GB on PND 42 and euthanized at 1, 6, 24 or 72 h post-exposure. The length of dendritic spines was signficantly (*, p<.05) shorter at the 24 h time point in the CA1 of rats exposed to 1.0 LD_{50} GB. Fewer and shorter dentritic spines were also observed at the 1 and 72 h time points in the BLA of rats exposed to 1.0 LD_{50} GB.

Key Research Accomplishments -

- Characterized the behavioral, physiological and neuropathological effects associated with sc exposure to GB in PND 7, 21 and 42 rats
- Evaluated seizure activity and dendritic spine density following sc exposure to GB in PND 42 rats

Conclusions -

Similar to adult models, juvenile rats (PND 42) exposed to 1.0 LD_{50} GB demonstrate motor control deficits and decreased ambulatory movement at 1 day post-exposure, as well as spatial memory impairments at 3 weeks post-exposure. Data suggest that these rats experience more spontaneous recurrent seizures compared to adult rats (data not shown), which may lead to extensive neuropathology as evidenced by a decrease in dendritic spine density in the basolateral amygdala of female rats exposed to 1.0 LD_{50} GB on PND 42. In addition, a small percentage of male rats exposed to 1.0 LD_{50} GB on PND 42 have elevated levels of troponin in their blood; however, this is not associated with myocardial damage. In contrast, few effects were observed in rats exposed to GB on PND 7 or 21. This study shows that nerve agent exposure during puberty results in severe and life-altering consequences in the rat model, which should be an area of further investigation.

Publications, Abstracts, and Presentations -

Wright LKM, Miller DB, Muse WT, Emm EJ, Lee RB, Whalley CE and Lumley LA (2014) Younger rats are more susceptible to the lethal effects of sarin than adult rats: 24 h LC_{50} for whole-body (60 min) exposure. The Toxicologist CD – An Official Journal of the Society of Toxicology 138:581i.

Wright L, Bourne A, Furman A, Stone M, Rossetti F, Lumley L (2014) Behavioral and neuropathological effects associated with subcutaneous exposure to sarin in juvenile rats. Neurotox Teratol 43:93.

Wright LKM, Whalley CE and Lumley LA (2014) Sarin-induced toxicity in young rats: assessment of lethality, behavior, physiology and neuropathology. 19th Biennial Medical Defense Bioscience Review. Hunt Valley, MD.

Inventions, Patents and Licenses -

Nothing to report

Reportable Outcomes -

Small animal model for exposure to sarin during development has been developed and characterized in terms of its physiological, behavioral and neuropathological effects. This model may be used in the future to evaluate medical countermeasures for developmental exposure to nerve agents.

Other Achievements -

Nothing to report

References -

de Araujo Furtado M, Zheng A, Sedigh-Sarvestani M, Lumley L, Lichtenstein S and Yourick D (2009) Analyzing large data sets acquired through telemetry from rats exposed to organophosphorus compounds: an EEG study. J Neurosci Methods. 184:176-83.

Appendices -

Personnel:

Name	*Role on Project*
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Andrew Bourne	Research Laboratory Technician III
Grace Calvin	Research Laboratory Technician II
Dilber Nurmemet	Research Lab Tech II
Michael Pham	Toxicologist
Dr. Franco Rossetti	Research Scientist
Sarah Sanjakdar	Post-Doctoral Fellow
Caroline Schultz	Research Laboratory Technician II
Mark Schultz	Research Associate
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